Discovery of Molecular Pathways Mediating 1,25-Dihydroxyvitamin D_3 Protection Against Cytokine-Induced Inflammation and Damage of Human and Male Mouse Islets of Langerhans

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Protection against insulitis and diabetes by active vitamin D, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), in nonobese diabetic mice has until now mainly been attributed to its immunomodulatory effects, but also protective effects of this hormone on inflammation-induced β-cell death have been reported. The aim of this study was to clarify the molecular mechanisms by which $1,25(OH)_2D_3$ contributes to β-cell protection against cytokine-induced β-cell dysfunction and death. Human and mouse islets were exposed to IL-1β and interferon-γ in the presence or absence of $1,25(OH)_2D_3$. Effects on insulin secretion and β-cell survival were analyzed by glucose-stimulated insulin release and electron microscopy or Hoechst/propidium iodide staining, respectively. Gene expression profiles were assessed by Affymetrix microarrays. Nuclear factor-κB activity was tested, whereas effects on secreted chemokines/cytokines were confirmed by ELISA and migration studies. Cytokine exposure caused a significant increase in β-cell apoptosis, which was almost completely prevented by $1,25(OH)_2D_3$. In addition, $1,25(OH)_2D_3$ restored insulin secretion from cytokine-exposed islets. Microarray analysis of murine islets revealed that the expression of approximately 4000 genes was affected by cytokines after 6 and 24 hours ($n = 4$; >1.3-fold; $P < .02$), of which nearly 250 genes were modified by $1,25(OH)_2D_3$. These genes belong to functional groups involved in immune response, chemotaxis, cell death, and pancreatic β-cell function/phenotype. In conclusion, these findings demonstrate a direct protective effect of $1,25(OH)_2D_3$ against inflammation-induced β-cell dysfunction and death in human and murine islets, with, in particular, alterations in chemokine production by the islets. These effects may contribute to the beneficial effects of $1,25(OH)_2D_3$ against the induction of autoimmune diabetes. **(*Endocrinology* 155: 736–747, 2014)**

Type 1 diabetes (T1D) is an autoimmune disease characterized by immune cell infiltration into the islets of Langerhans and a progressive destruction of the insulin-secreting β-cells. This autoimmune attack is mediated by the release of proinflammatory cytokines, such as IL-1β, interferon-γ (IFN-γ), and TNF-α, along with the production of other cytotoxic factors, such as reactive oxygen and nitrogen species, and direct cell killing involving Fas re-

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Abbreviations: CCL, chemokine (C-C motif) ligand; CXCL, chemokine (C-X-C motif) ligand; GSIS, glucose-stimulated insulin secretion; IFN-γ, interferon γ; IPA, immunoprecipitation assay; IκB, inhibitor of NF-κB; NF-κB, nuclear factor-kappa B; NOD, nonobese diabetic; NOD.SCID, non-obese diabetic severe combined immune deficiency; $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; qRT-PCR, quantitative real-time PCR; T1D, type 1 diabetes; VDR, vitamin D receptor.

ceptor/Fas ligand and perforin/granzyme (1, 2). Previous in vitro studies have demonstrated that exposure of human, mouse, and rat islets, as well as clonal β-cell lines, to a combination of cytokines triggers functional impairment and cell death (3). Cytokine exposure induces β-cell apoptosis by activation of signaling pathways, mainly controlled by the transcription factors nuclear factor κB (NF-κB) and signal transducer and activator of transcription-1 (STAT-1). This, in turn, leads to nitric oxide production, endoplasmic reticulum stress, and increased chemokine production resulting in increased local inflammation and progressive β-cell death (1).

Aside from its long-known role in calcium/phosphate homeostasis and bone health, the active form of vitamin D, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), has been identified as a modulator of the immune system playing a significant role in the defense against autoimmune diseases including T1D (4, 5). In support of this, vitamin D deficiency has been linked to an increased incidence of T1D (6, 7), whereas supplementation with vitamin D prevents or delays the onset of T1D (unpublished personal communication with T. Takiishi). The ubiquitous expression of the receptor for vitamin D (VDR) includes β-cells (8), and some evidence exists that $1,25(OH)_2D_3$ exerts direct protective effects on the β-cell (9). Whether or not $1,25(OH)_2D_3$ is capable of ameliorating in vitro cytokine-impaired insulin release and protection against cytokine-induced cell death remains controversial (10–14). Attempts have already been made to identify the mechanisms responsible for a potential protection of islet function and survival by $1,25(OH)_2D_3$ (9). There are no studies, however, aiming at clarifying the global mechanisms behind these beneficial effects in pancreatic β-cells.

We have addressed this question by investigating the protective effects of $1,25(OH)_2D_3$ on β-cell function and survival in human and mouse islets. For further in-depth analysis of the pathways involved, we performed Affymetrix microarray and immunoprecipitation assay (IPA) analysis to identify global gene expression changes induced by $1,25(OH)_2D_3$ in mouse pancreatic islets exposed to IL-1β and IFN-γ. We investigated in detail the genes and gene networks affected by the proinflammatory cytokines and identified restorative effects of $1,25(OH)_2D_3$ on different functional groups such as chemokines, NF-κB-related genes, metabolic genes, and β-cell transcription factors. These alterations in gene expression profile correlate with the observed protective effects on β-cell function and survival, showing that $1,25(OH)_2D_3$ has important direct protective effects on β-cells in an inflammatory setting. These findings, together with the well-known effects of $1,25(OH)_2D_3$ as an immunomodulatory agent, provide a rationale for the use of $1,25(OH)_2D_3$ or analogs in the prevention or treatment of T1D.

Materials and Methods

The methods for chemotaxis experiments and NF-κB activity assay are provided as Supplemental Data published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org.

Preparation and culture of human islets

Human islets were prepared by enzymatic digestion and density gradient purification (15, 16) from pancreata of 6 multiorgan donors (age 65 ± 21 years; body mass index, 26.3 ± 5.4 kg/m^2) and cultured in M199 medium containing 5.5 mM glucose, supplemented with 10% (vol/vol) serum and 100 U/mL penicillin, 100 μg/mL streptomycin, 50 μg/mL gentamicin, and 750 ng/mL amphotericin B (Sigma-Aldrich) (15, 16). The islets were incubated for 24 hours either with or without 10^{-8}M $1,25(OH)_2D_3$ (Sigma-Aldrich) and subsequently cultured for 48 hours with 50 U/mL IL-1β and 1000 U/mL IFN-γ (Roche Diagnostics S.p.A.) with or without 10^{-8}M $1,25(OH)_2D_3$.

Isolation and culture of mouse pancreatic islets

Islets were isolated from 2- to 3-week-old male C57BL/6 mice (Harlan) as previously described (17) and exposed for 6 and 24 hours to 4 different conditions: 1) vehicle (0.000625% ethanol); 2) $1,25(OH)_2D_3$ (10^{-8} M); 3) IL-1β (50 U/mL), IFN-γ (100 U/mL) and vehicle; and 4) $1,25(OH)_2D_3$, IL-1β and IFN-γ. Human IL-1β was a gift from Dr. C. W. Reinolds, National Cancer Institute, National Institutes of Health, Bethesda, MD. Murine IFN-γ and $1,25(OH)_2D_3$ were obtained from Peprotech and Sigma-Aldrich, respectively. All animal experiments were approved by the Ethics Commision of the KU Leuven.

Human islet cell death and ultrastructure analysis

Assessment of human islet cell death and ultrastructure analysis were performed by electron microscopy studies as described previously (18, 19). Briefly, cells were considered dead on the basis of any of the following criteria: Loss of plasma membrane integrity, fragmentation into discrete bodies, and engulfment of cell corpse or its fragments by an adjacent cell. The presence of marked chromatin condensation and/or blebs was considered as an indication of apoptosis. For morphometric studies, 25 micrographs, obtained at ×10 000, were analyzed by overlay with a graticule (11 × 11 cm) composed of 169 points. For the study of insulin granules, volume density (VD), expressed as milliliters/100 mL tissue (ml%), was calculated according to the formula: VD = Pi/Pt, where Pi is the number of points within the subcellular component, and Pt is the total number of points.

Determination of murine islet cell death and rat INS-1E cells

Murine islets were exposed to cytokines for 6 and 24 hours in the presence or absence of $1,25(OH)_2D_3$ as described earlier (*Isolation and culture of mouse pancreatic islets*). INS-1E cells (a kind gift from Professor C. Wollheim, Centre Medical Universitaire, Geneva, Switzerland), were exposed to cytokines for 24

hours (10 U IL-1β + 100 U IFN-γ), in the presence or absence of $1,25(OH)_2D_3$ (10^{-8}M) either coincubated or preincubated for 24 hours. Following 20 minutes of incubation with 50 μg/mL Hoechst 33342 and 5 μg/mL propidium iodide (Life Technologies) at 37°C, the percentage of apoptosis in each islet was estimated or 500 INS-1E cells were counted on an inverted fluorescence microscope by two observers, one of them being unaware of sample identity.

Glucose-stimulated insulin secretion from human islets

For insulin secretion studies of human islets, which were performed as previously detailed (18, 19), islets were first kept at 37°C for 45 minutes in Krebs-Ringer bicarbonate, 0.5% (vol/vol) albumin, pH 7.4, containing 3.3 mM glucose (wash-out phase). Next, the medium was replaced with Krebs-Ringer bicarbonate containing 3.3 mM glucose to assess basal secretion for 45 minutes followed by a further 45-minute incubation with 16.7 mM glucose to assess insulin response to acute challenge. Insulin was quantified using an immunoradiometric assay (Pantec Forniture Biomediche).

Glucose-stimulated insulin secretion from murine islets

After 24 hours of culture in medium supplemented with cytokines in the presence or absence of $1,25(OH)_2D_3$, 15–20 islets were washed 3 times in glucose-free HEPES-Krebs buffer and incubated at 37°C and 5% CO_2 for 30 minutes, after which part of the supernatant was stored at −20°C for further analysis, and islets were incubated for another 2 hours in HEPES-Krebs buffer containing 3 or 30 mM glucose at 37°C and 5% CO_2. Supernatants were stored at −20°C. The islets were washed 3 times in ice-cold PBS and placed in a 1:2 mix of ice-cold PBS and extraction buffer (182 mM H_3PO_4, 83% EtOH) overnight at 4°C. Then, the islets were sonicated and centrifuged at 3500 rpm for 10 minutes, and supernatants were stored at −20°C. Insulin concentrations were measured with a commercial insulin ELISA kit (Mercodia) on the Victor^3 1420 Multilabel Counter (PerkinElmer). Final values of glucose-stimulated insulin secretion (GSIS) were calculated as insulin released over 2 hours, divided by the total insulin content.

Chemokine and cytokine secretion

The concentrations of secreted chemokines and cytokines in culture supernatants of murine islets were determined by either ELISA (R&D Systems) or FlowCytomix assay (eBioscience) according to the manufacturer's instructions.

RNA isolation, reverse transcription, and quantitative real-time PCR (qRT-PCR)

Total RNA from murine islets was extracted using the RNeasy Micro Kit (Qiagen) and from human islets using the PureLink RNA mini Kit (Life Technologies). cDNA synthesis for mouse and human islets was done using SuperScript II Reverse Transcriptase (Invitrogen) or the iScript cDNA Synthesis Kit (Bio-Rad Laboratories), respectively. qRT-PCR was performed with gene-specific forward and reverse primers using either Fast SYBR Green Master Mix or a gene-specific TaqMan probe in combination with TaqMan Fast Universal Master Mix (Life Technologies). Samples were analyzed on the StepOnePlus Real-Time PCR System (Life Technologies). Relative RNA expression was obtained by means of the comparative cycle threshold (Ct) method after normalization to the geometrical mean of Ct values for ribosomal protein L27 (*Rpl27*), hypoxanthine guanine phosphoribosyl transferase (*Hprt*), and β-actin.

Microarray and pathway analysis

Total RNA was isolated as described previously (*RNA isolation, reverse transcription and qRT-PCR*). RNA quality was examined by the 2100 Bioanalyzer (Agilent), and samples with an RNA integrity number above 7.5 were accepted for microarray analysis. Amplified sense-strand DNA was generated using the Affymetrix GeneChip Whole Transcript Sense Target Labeling Assay according to the manufacturer's instructions (Affymetrix). The incorporation of dUTP into the final sense-strand DNA allowed for fragmentation and subsequent biotin labeling. Labeled DNA fragments were hybridized overnight to Affymetrix GeneChip Mouse Gene 1.0 ST Arrays. The arrays were washed and stained with streptavidin-phycoerythrin on an automated Affymetrix Fluidics Station and scanned using the Affymetrix 3000 GeneScanner. Microarray data were evaluated by manual investigation as well as by the use of IPA software.

Statistics

The ANOVA test with Bonferroni correction or the paired Student's *t* test were applied for the in vitro experiments on mouse and human islets. $P < .05$ was considered as significant. For age and body mass index of human donors, data are presented as means ± SD. For all other experiments, data are presented as means ± SEM. Microarray image files were analyzed using the Affymetrix command console software, and the robust multichip average (RMA) algorithm was used to calculate the signal intensities for each probe cell. Detection above background calls (absent, marginal, or present) were determined by the XPS Bioconductor package (http://www.bioconductor.org/packages/release/bioc/html/xps.html), and only probe sets declared "present" in at least 50% of the replicates (2 of 4) in at least 1 condition and 1 time point were considered for further analysis. A list of differentially expressed genes was generated for each treatment group with the Welch's *t* test using cutoff values of $P < .02$ and at least a 1.3 median fold change between treated samples and respective controls. For IPA analysis (Ingenuity Systems, Inc.), statistically significant canonical pathways and cellular functions were identified at $P < .05$ by Fisher's exact test.

Results

Effect of $1,25(OH)_2D_3$ on cytokine-induced islet cell death and impaired glucose-stimulated insulin release

We examined the effect of $1,25(OH)_2D_3$ on the viability of cytokine-exposed human and murine islets. As expected, electron microscopy revealed a significant increase in apoptotic death after exposure of human islets to IL-1β + IFN-γ for 48 hours (from 1.0% to 11.0%; n ≥ 250 cells

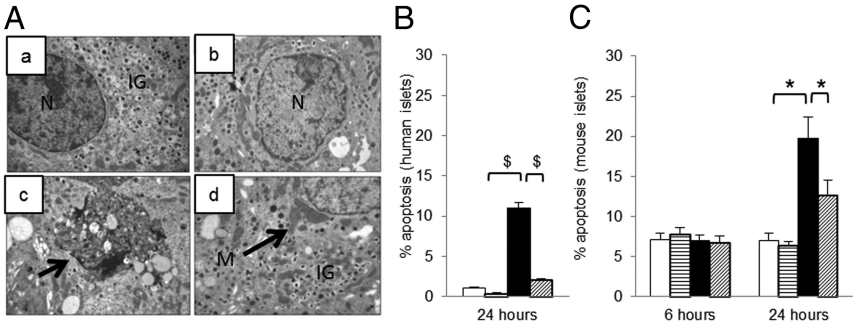

Figure 1. A and B, Prevention of apoptosis by $1,25(OH)_2D_3$ in cytokine-exposed human islets, as determined by electron microscopy. A, Representative images of human islets precultured for 24 hours with or without $1,25(OH)_2D_3$ followed by 48 hours of exposure to cytokines and/or $1,25(OH)_2D_3$. a, Control; b, 10^{-8} M $1,25(OH)_2D_3$; c, 50 U/mL IL-1β and 1000 U/mL IFN-γ; and d, 10^{-8} M $1,25(OH)_2D_3$, 50 U/ml IL-1β and 1000 U/mL IFN-γ. The black arrow in panel c indicates an apoptotic β-cell in a sample treated with cytokines; N, nucleus; IG, insulin granules; M, mitochondria. Based on this electron microscopy the percentage of apoptotic β-cells was determined in human islets (B). Open bar, control medium; shaded bar, 10^{-8} M $1,25(OH)_2D_3$; solid bar, 50 U/mL IL-1β and 1000 U/mL IFN-γ; and hatched bar, 10^{-8} M $1,25(OH)_2D_3$, 50 U/mL IL-1β and 1000 U/mL IFN-γ. Data are presented as means ± SEM (n = 6 islets, $^{\$}$, $P < .05$ vs all groups and vs cotreatment with $1,25(OH)_2D_3$, Bonferroni corrected). C, Estimated % of apoptotic cells in $1,25(OH)_2D_3$- and cytokine-exposed murine islets determined by Hoechst-PI staining. Islets were cultured for 6 and 24 hours in open bar, control medium; shaded bar, 10^{-8} M $1,25(OH)_2D_3$; solid bar, 50 U/mL IL-1β and 100 U/mL IFN-γ; and hatched bar, 10^{-8} M $1,25(OH)_2D_3$, 50 U/mL IL-1β and 100 U/mL IFN-γ. Data are presented as means ± SEM (n = 10 independent experiments, *, $P < .001$).

per condition; $P < .05$) (Figure 1, A and B). $1,25(OH)_2D_3$ prevented, to a large extent, this deleterious effect of cytokines, with a proportion of apoptotic β-cells of only 2.0% after addition of $1,25(OH)_2D_3$ to the cytokine-treated islet cultures ($P < .05$). Similarly, an increased cell death (2.8-fold) was observed in murine islets treated with IL-1β + IFN-γ for 24 hours (19.7% with cytokine treatment vs 7.0% in controls; n = 10; $P < .001$). Again, cotreatment of islets with $1,25(OH)_2D_3$ partially reversed the induction of apoptosis (to 12.6%) ($P < .001$; n = 10) (Figure 1C). Longer exposure, ie, 48 and 72 hours, revealed the same pattern of partial protection (data not shown). Of note, treatment of rat insulin-producing INS-1E cells for 24 hours with IL-1β + IFN-γ, either preincubated for 24 hours or coincubated with $1,25(OH)_2D_3$, did not protect INS-1E cells against cytokine-mediated cell death (data not shown), confirming previously published findings in INS-1E cells and purified rat β-cells (10).

Next, we investigated the effect of cytokine exposure on GSIS and the potentially protective effect of $1,25(OH)_2D_3$ thereon. Basal insulin release in human islets (at 3.3 mM glucose) was 59.5 μU/mL and increased to 140 μU/mL ($P < .05$; n = 6) at 16.7 mM glucose, corresponding to a stimulation index of 2.4. GSIS (expressed as stimulation index) decreased significantly upon cytokine exposure (72% of control islets; $P < .05$; n = 6), and returned to similar levels to that of control islets in the presence of $1,25(OH)_2D_3$ (99%; n = 6) (Figure 2A). Volume density of insulin granules (mL%) (Figure 2B) differed between the experimental conditions ($P < .01$ by ANOVA) and was 6.3 in control β-cells, 6.7 in $1,25(OH)_2D_3$-exposed cells, 3.0 with cytokines ($P < .05$ vs the other groups), and 6.7 in the presence of both $1,25(OH)_2D_3$ and cytokines. These data were supported by similar observations in mouse islets. Preexposure of murine islets for 24 hours to IL-1β and IFN-γ significantly decreased insulin release during 2 hours of glucose stimulation (30 mM) (52% of control level; $P < .05$; n = 7). This effect was completely restored by coculturing islets with $1,25(OH)_2D_3$ for 24 hours prior to GSIS ($P < .05$; n = 7) (Figure 3). $1,25(OH)_2D_3$ alone did not affect insulin secretion in either murine (Figure 3) or human islets (Figure 2A).

Gene networks regulated by IL-1β and IFN-γ in murine islets of Langerhans

Exposure to IL-1β + IFN-γ resulted in 3814 and 4439 differentially expressed genes after 6 and 24 hours, respectively. Using IPA the most significant molecular and cellular functions (Supplemental Figure 1) as well as canonical pathways (Supplemental Figure 2) were identified. Manual analysis was performed in parallel (by D.L.E., Supplemental Table 1). Several genes associated with

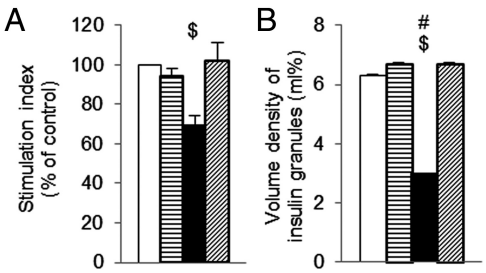

Figure 2. Effect of $1,25(OH)_2D_3$ on glucose-stimulated insulin release and volume density of insulin granules in human islets exposed to IL-1β and IFN-γ Islets were precultured for 24 hours with $1,25(OH)_2D_3$ or control medium followed by 48 hours of exposure to cytokines in the presence or absence of $1,25(OH)_2D_3$. A, Stimulation index and B, volume density of insulin granules (mL%) of human islets subjected to the following: open bar, control medium; shaded bar, 10^{-8} M $1,25(OH)_2D_3$; solid bar, 50 U/mL IL-1β and 1000 U/mL IFN-γ; and hatched bar, 10^{-8} M $1,25(OH)_2D_3$, 50 U/mL IL-1β, and 1000 U/mL IFN-γ. Data are presented as means ± SEM (A: n = 6 islets, *, $P < .05$ vs control, $1,25(OH)_2D_3$ and $1,25(OH)_2D_3$ + cytokines (Bonferroni corrected); B, n > 250 cells studied per condition; $^{\$}$, $P < .05$ vs all other conditions).

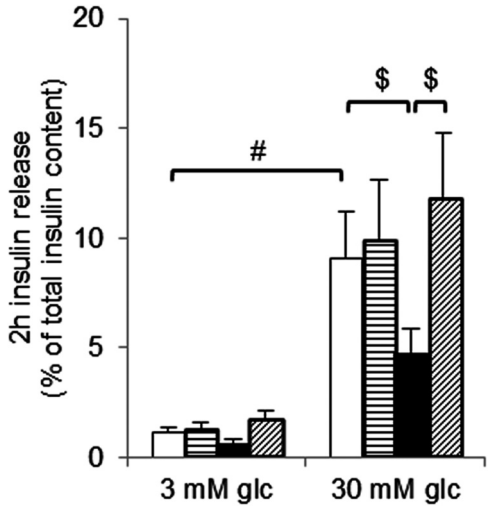

Figure 3. Effect of 1,25(OH)$_2$D$_3$ on glucose-stimulated insulin secretion (GSIS) (% of content) by isolated cytokine-exposed mouse islets Prior to GSIS (consisting of 2 hours of incubation at 3 and 30 mM glucose [glc]), islets were subjected to 24 hours of in vitro culture in the following: open bar, control medium or medium containing shaded bar, 10^{-8} M 1,25(OH)$_2$D$_3$; solid bar, 50 U/mL IL-1β and 100 U/mL IFN-γ; or hatched bar, 10^{-8} M 1,25(OH)$_2$D$_3$, 50 U/mL IL-1β and 100 U/mL IFN-γ. Data are presented as means ± SEM (n=7 independent experiments; #, $P < .01$; $, $P < .05$).

NF-κB and IFN-γ signaling were induced after 6 and 24 hours of cytokine exposure. A significant immune response was evident by the induction of a high number of proteasome subunits, HLA-related molecules, a clear antiviral response, and an increase in the expression of a multitude of chemokines, cytokines, and adhesion molecules. IL-1β and IFN-γ modified the expression of genes related to endoplasmic reticulum stress and caused an up-regulation of apoptotic genes including several caspases, whereas genes related to DNA damage and free radical scavenger response were also differentially expressed. On the other hand, there was an overall decrease in the expression of genes involved in cell cycle as well as glycolysis, Krebs cycle, and lipid metabolism. A similar expression pattern (ie, decreased expression) was detected for several important β-cell transcription factors and various hormone and growth factor receptors.

Effects of 1,25(OH)$_2$D$_3$ on gene pathways regulated by IL-1β and IFN-γ in murine islets of Langerhans

Microarray analysis allowed for identification of genes that were differentially expressed after exposure of islets to 1,25(OH)$_2$D$_3$ in combination withIL-1β and IFN-γ as compared with cytokine exposure alone. Only genes for which the expression levels were also altered by cytokines vs controls were considered. In total, 119 and 243 genes were differentially expressed after 6 and 24 hours, respectively, when these conditions were compared. Top scoring molecular and cellular functions generated by IPA (Supplemental Figure 3) included genes related to cellular movement (ie, chemotaxis), cellular assembly and organization, cellular function and maintenance, and cellular development as well as cellular growth and proliferation. Cell death, carbohydrate and lipid metabolism, cell-to-cell signaling and interaction, vitamin metabolism and molecular transport were also affected. Because these terms are rather general and do not take into account the cell type in question, manual classification was carried out in parallel (by H.W.-K.) as described in the following sections.

Among the genes that were induced by cytokine treatment, 38 and 74 genes were fully or partially normalized after 6 and 24 hours of cotreatment with 1,25(OH)$_2$D$_3$, respectively. The main gene classes were immune response genes such as chemokines and cytokines as well as genes involved in NF-κB signaling. 1,25(OH)$_2$D$_3$ also partially or fully reversed the cytokine-induced down-regulation of 37 and 110 genes after 6 and 24 hours of exposure, respectively. This group included genes associated with metabolism, transport and homeostasis of ions and other substances, as well as transcription factors involved in the maintenance of differentiated β-cell functions. Furthermore, genes involved in intracellular trafficking, exocytosis, and cytoskeletal organization were differentially expressed after exposure of cytokine-treated islets to 1,25(OH)$_2$D$_3$. Of note, genes involved in vitamin D metabolism and signaling including *Vdr* and vitamin D-binding protein (*DBP/Gc*) were clearly expressed and the well-known 1,25(OH)$_2$D$_3$-target gene, *Cyp24a1*, was significantly up-regulated, confirming direct 1,25(OH)$_2$D$_3$ effects (Table 1 and Supplemental Table 2).

Effect of 1,25(OH)$_2$D$_3$ on IL-1β and IFN-γ-induced expression of genes involved in NF-κB signaling and activity

Most NF-κB family members and regulatory genes were up-regulated after both 6 and 24 hours of cytokine treatment (Supplemental Table 1). Among the most highly induced genes were the NF-κB subunit nuclear factor of κ-light polypeptide gene enhancer in B-cells 2 (*Nfkb2/p52*) and inhibitor of κB kinase ϵ (*Ikbke/IKKϵ*), a gene encoding an IκB kinase that mediates inactivation of NF-κB inhibitors. Interestingly, a partial normalization was observed after 24 hours of cotreatment with 1,25(OH)$_2$D$_3$ for *p52*, *IKKϵ* , and the classical NF-κB inhibitors *Nfkbib/IκBβ* and *Nfkbie/IκBϵ* (Table 1 and Supplemental Table 2). In line with these findings, whole-cell extracts isolated from islets after 30 minutes of cytokine exposure showed an increase in p52-specific binding

Table 1. Selected genes differentially regulated by treatment with 10^{-8} M 1,25(OH)$_2$D$_3$, cytokines (50 U/mL IL-1β and 100 U/mL IFN-γ), or the combination in murine pancreatic islets. Data are expressed as fold change vs respective control (n = 4 independent experiments)

		6 hour (fold change)			24 hour (fold change)		
		Cyt vs Con	1.25+Cyt vs Cyt	1.25 vs Con	Cyt vs Con	1.25+Cyt vs Cyt	1.25 vs Con
Symbol	Gene name/functional group						
Vitamin D metabolism and signaling							
Cyp24a1	Cytochrome P450, family 24, subfamily a, polypeptide 1	2.74[a]	56.2[a]	98.19[a]	−1.05	119.02[a]	88.95[a]
Vdr	Vitamin D receptor	−1.56[b]	1.06	−1.19	−2.25[b]	−1.01	−1.08
Gc/DBP	Group-specific component/ vitamin D-binding protein	−1.13	−1.02	−1.09	−1.27	−1.2	−1.24
NF-κB signaling							
Ikbke (IKKε)	Inhibitor of κB kinase ε	9.22[a]	−1.1	1.13	2.99[a]	−1.31[b]	1.05
Nfkb2 (p52)	Nuclear factor of κ light polypeptide gene enhancer in B-cells 2, p49/p100	10.76[a]	−1.13	1.09	3.86[a]	−1.81[a]	−1.07
Nfkbib (IκBβ)	Nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, β	2.7[a]	−1.07	−1.06	1.95[a]	−1.26[b]	−1
Nfkbie (IκBε)	Nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, ε	3.26[a]	−1.39[a]	1.08	1.49[d]	−1.56[b]	−1.08
Chemokines and cytokines							
Ccl2 (MCP-1)	Chemokine (C-C motif) ligand 2	70.4[a]	−1.55[b]	2.13	15.19[a]	−1.96[c]	−1.01
Ccl5 (RANTES)	Chemokine (C-C motif) ligand 5	3.99[a]	−1.67[b]	1.13[d]	4.21[a]	−1.6	1.04
Ccl8 (MCP-2)	Chemokine (C-C motif) ligand 8	2.15[a]	−1.44[d]	1.04	3[a]	−1.66[b]	1.04
Cxcl1	Chemokine (C-X-C motif) ligand 1	14.57[a]	−1.4[b]	1.24	4.06[a]	−1.35[b]	1
Cxcl11	Chemokine (C-X-C motif) ligand 11	43.34[a]	−1.4[b]	1.42	13.57[a]	−3[a]	1.05
Cxcl16	Chemokine (C-X-C motif) ligand 16	6.24[a]	−1.3[b]	−1.03	8.57[a]	−1.4[d]	−1
Cxcl2	Chemokine (C-X-C motif) ligand 2	23.18[a]	−1.9[b]	1.1	2.78[d]	−1.25	−1.17
Cxcl9	Chemokine (C-X-C motif) ligand 9	203.3[a]	−1,17	2.2	191.01[a]	−1.74[d]	1
Il15	IL-15	2.55[a]	−1.42[b]	−1.1[b]	1.64[c]	−1.31[d]	1.01
Il18	IL-18	1.7[a]	−1.44[b]	−1.04	−1.03	1.02	−1.04
Il6	IL-6	26.26[a]	−1.49[d]	1.16	14.77[a]	−1.56	1.02
Transcription factors							
Dll1	δ-like 1 (*Drosophila*)	3.99[a]	−1.39[c]	−1.04	1.98[b]	−1.36[d]	−1.14[d]
Hes1	Hairy and enhancer of split 1 (*Drosophila*)	−1.39[b]	1.12	−1.19[d]	1.6[c]	−1.41[c]	−1.01
Id2	Inhibitor of DNA binding 2	2.11[a]	−1.34[b]	−1.26[b]	2.01[b]	−1.22	−1.17
Isl1	ISL1 transcription factor, LIM/homeodomain	−1.53[a]	−1.04	−1.06	−3.63[a]	1.3[d]	−1.03
Myc	Myelocytomatosis oncogene	1.54[b]	−1.31[b]	−1.33[d]	4.74[b]	1.04	−1
Neurod1	Neurogenic differentiation 1	−1.12	1.22[b]	1.04	−2.15[b]	1.43[b]	1.09[b]
Pax6	Paired box gene 6	−1.35[a]	1.06	1.1	−2.4[a]	1.3[b]	−1.02
Glucose metabolism							
Gck	Glucokinase	1.05	1.23[c]	1.04	−1.46[b]	1.32[b]	1.07
Idh1	Isocitrate dehydrogenase1 (NADP+), soluble	−1.49[a]	1.37[a]	1.26[d]	−2.42[a]	1.78[b]	1.05
Ldha	Lactate dehydrogenase A	1.11	−1.01	−1.06	1.94[c]	−1.41[c]	1.09
Nubpl	Nucleotide binding protein-like	−1.08	−1.15	−1.15[b]	−1.32[c]	1.43[c]	1.02
Pdha1	Pyruvate dehydrogenase E1 α 1	−1.92[b]	1.3[b]	1.02	−3.46[a]	1.83[a]	1.25[b]
Others							
Nos2 (iNOS)	Nitric oxide synthase 2, inducible	148.83[a]	−1.27[b]	1.76	40.93[a]	−1.26	1.17

This list is a short version of Supplemental Table 2 including genes of particular interest for β-cells in the setting of T1D. Con, Control; 1,25, 1,25(OH)$_2$D$_3$; Cyt, IL-1β and IFN-γ.

[a], $P < .001$; [b], $P < .01$; [c], $P < .02$; [d], $P < .05$ by Welch's *t* test.

to oligos containing a NF-κB consensus binding site (1.64 fold; $P < .015$). This binding activity was detected by an antibody specific for active p52. Pretreatment (24 hours) with 1,25(OH)$_2$D$_3$ reduced the p52-specific DNA-binding activity by 47% (−1.26 fold; $P < .05$) (Figure 4).

Effect of 1,25(OH)$_2$D$_3$ on IL-1β and IFN-γ-induced expression of immune response genes and chemotaxis

Genes related to chemotaxis and cytokine signaling constituted the major cytokine-induced group partially

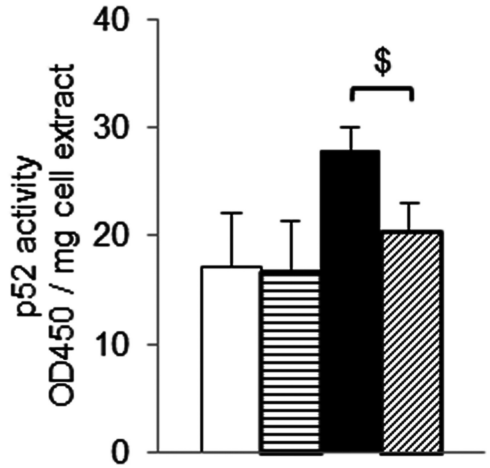

Figure 4. Effect of 1,25(OH)$_2$D$_3$ on NF-κB2/p52 binding activity of cytokine-exposed murine islets Binding activity (OD450) was measured per mg whole-cell extract obtained from islets precultured for 24 hours in the presence or absence of 10^{-8} M 1,25(OH)$_2$D$_3$ followed by 30 minutes of culture with or without 50 U/mL IL-1β and 100 U/mL IFN-γ. Open bar, control; shaded bar, 10^{-8} M 1,25(OH)$_2$D$_3$; solid bar, 50 U/mL IL-1β and 100 U/mL IFN-γ; and hatched bar, 10^{-8} M 1,25(OH)$_2$D$_3$, 50 U/mL IL-1β, and 100 U/mL IFN-γ. Data are presented as means ± SEM (n = 4 independent experiments, $, $P < .05$).

reversed by cotreatment with 1,25(OH)$_2$D$_3$ (Table 1 and Supplemental Table 2). The affected genes included inflammatory chemokines known to attract monocytes, neutrophils, or activated T cells and natural killer cells (20), ie, chemokine C-C motif ligand 2 (CCL2/MCP-1), 5 (CCL5/RANTES), and 8 (CCL8/MCP-2), chemokine C-X-C motif ligand 1 (CXCL1/KC), 2 (CXCL2/MIP-2α), 9 (CXCL9), 11 (CXCL11/I-TAC/IP9), and 16 (CXCL16), as well as the proinflammatory cytokines IL-6, IL-15, and IL-18. For several of these chemokines and cytokines this regulatory pattern was confirmed by qRT-PCR (Figure 5A) and paralleled by an altered protein secretion (Figure 5B). In general, the effects of 1,25(OH)$_2$D$_3$ on the expression of these genes were more marked after 6 hours, suggesting an early and specific action. Interestingly, most of the alterations in chemokine mRNA expression, by IL-1β and IFNγ, as well as the inhibitory effect of 1,25(OH)$_2$D$_3$ thereon, could be confirmed in human islets, measured after 24 hours of treatment. Indeed, similar trends were observed at the RNA level for the chemokines CXCL9, CXCL10, and CXCL11 as well as for the cytokines IL-6 and IL-15 (Supplemental Figure 4).

We further investigated whether exposure of islets to 1,25(OH)$_2$D$_3$ could inhibit the migration of elicited monocytes toward cytokine-conditioned islet supernatant (n = 7; Figure 6). Following 3 days of exposure to cytokines, the migration of monocytes increased significantly (1.55-fold compared with control; $P < .02$), whereas this migration was significantly lower for cotreatment with 1,25(OH)$_2$D$_3$, as compared with cytokine exposure alone (−1.28-fold; $P < .01$).

Effect of 1,25(OH)$_2$D$_3$ on cytokine-impaired expression of genes involved in maintenance of a differentiated β-cell function

Generally, the genes involved in development, differentiation, and function of β-cells displayed an early (6 hours) and sustained (24 hours) decrease following cytokine treatment (Supplemental Table 1). The transcription factors, paired box gene 6 (*Pax6*), neurogenic differentiation 1 (*NeuroD1*), and the LIM homeodomain transcription factor *Isl1*, all displayed reduced expression levels upon cytokine treatment. These effects were partially reversed by 1,25(OH)$_2$D$_3$ after 24 hours. The effect on *Pax6* and *Isl1* was confirmed by qRT-PCR (Figure 7). Moreover, we detected an induction of the notch ligand δ-like 1 (*Dll1*) as well as the downstream target gene, hairy and enhancer of split 1 (*Hes1*), by IL-1β and IFN-γ. However, this was partially prevented upon cotreatment with 1,25(OH)$_2$D$_3$. These effects of cytokines and cotreatment with 1,25(OH)$_2$D$_3$ were observed at both time points for *Dll1* but at 24 hours only for *Hes1*. Myelocytomatosis oncogene (*Myc*) and the target gene inhibitor of DNA binding 2 (*Id2*) displayed increased expression levels following exposure to cytokines at 6 and 24 hours, whereas 1,25(OH)$_2$D$_3$ decreased the effect of the cytokines at 6 hours only for both targets, suggesting that the beneficial effects of 1,25(OH)$_2$D$_3$ may be transitory in this context. The effect on *Myc* expression was confirmed by qRT-PCR (Figure 7).

Glucokinase (*Gck*), as well as *Pdha1*, encoding a component of the pyruvate dehydrogenase complex, were both down-regulated following 24 hours of cytokine treatment, but expression levels were fully and partially restored, after cotreatment with 1,25(OH)$_2$D$_3$, respectively, a finding confirmed by qRT-PCR for *Gck* (Figure 7). A similar effect was observed for isocitrate dehydrogenase 1 (*Idh1*), an enzyme catalyzing a pyruvate-cycling pathway, as well as nucleotide binding protein-like (*Nubpl*), which is required for assembly of the mitochondrial membrane respiratory chain reduced nicotinamide adenine dinucleotide dehydrogenase (Complex I). The opposite pattern was detected for lactate dehydrogenase (*Ldha*).

Discussion

In this study we investigated the protective actions of 1,25(OH)$_2$D$_3$ on islets of Langerhans in an inflammatory setting, relevant for T1D, and the molecular pathways

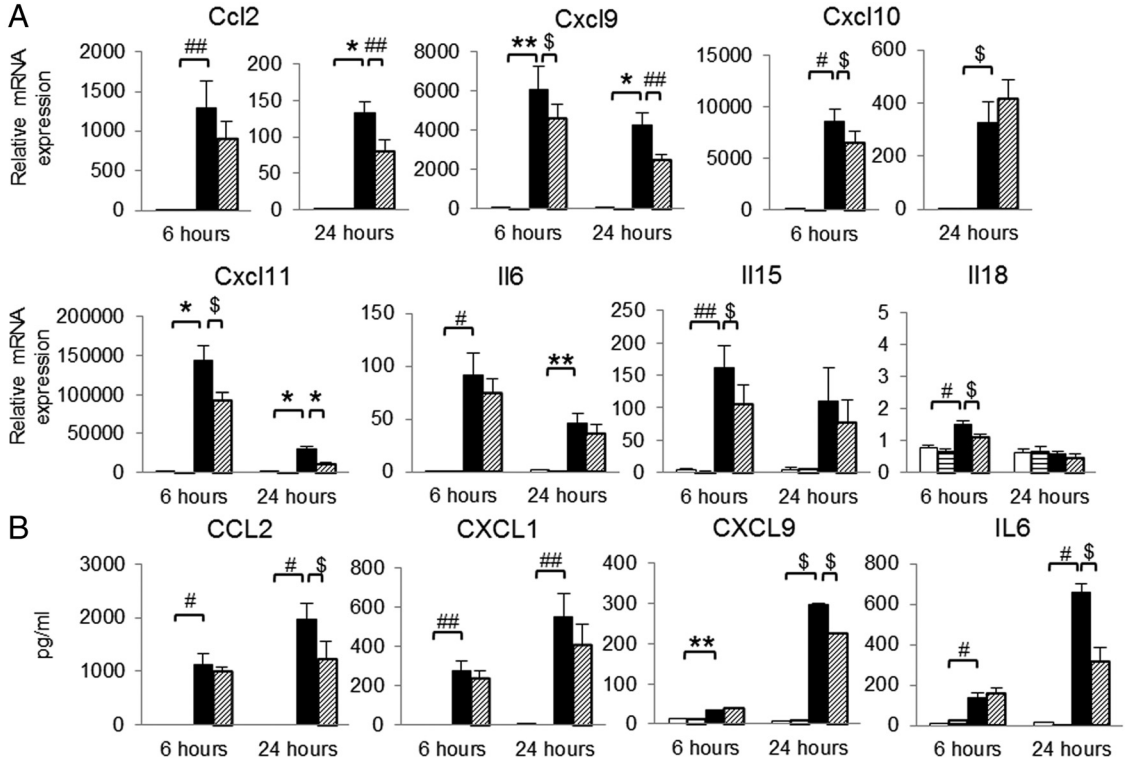

Figure 5. Effect of $1,25(OH)_2D_3$ on mRNA and protein expression of chemokines and cytokines in IL-1β- and IFN-γ-treated murine islets. Islets were cultured for 6 and 24 hours with the following: open bar, control medium; shaded bar, 10^{-8} M $1,25(OH)_2D_3$; solid bar, 50 U/mL IL-1β and 100 U/mL IFN-γ; and hatched bar, 10^{-8} M $1,25(OH)_2D_3$, 50 U/mL IL-1β, and 100 U/mL IFN-γ. A, mRNA expression determined by qRT-PCR and B, protein secretion assessed by ELISA. Data are presented as means ± SEM (n=4–7 independent experiments; *, $P < .001$; **, $P < .002$; #, $P < .01$; ##, $P < 0.02$; $, $P < 0.05$).

involved. Our data show that $1,25(OH)_2D_3$ protects both human and mouse islets against IL-1β and IFN-γ-induced apoptosis. This confirms previous reports by Riachy et al (12, 13), showing protection of human islets and the rat insulinoma RINm5F cell line against cytokine-mediated apoptosis by $1,25(OH)_2D_3$, but contrasts with our own observations where we did not find protection against apoptosis induced by cytokines in fluorescence-activated cell sorting-purified rat β-cells and INS-1E cells by in vitro exposure to $1,25(OH)_2D_3$, nor in islets derived from in vivo $1,25(OH)_2D_3$-treated NOD.SCID (nonobese diabetic severe combined immune deficiency) mice (10). These differences may be explained by differences in species (mouse and human vs rat) and by differences in experimental conditions (eg, in vitro vs in vivo exposure to $1,25(OH)_2D_3$), but not by differences due to pre- vs coexposure to $1,25(OH)_2D_3$ (when testing pre- or coexposure, the differences between human/mouse on one side vs rat on the other remained) or to different concentrations of cytokines (in this study as well as in the previous one from our group, the cytokine mixture was optimized for mouse-, human-, or rat-derived cells). Of particular interest, we show that $1,25(OH)_2D_3$ not only restores β-cell apoptosis, but also β-cell function in an inflammatory setting, both in murine and human islets. These observations in human tissue are of particular relevance for the human disease.

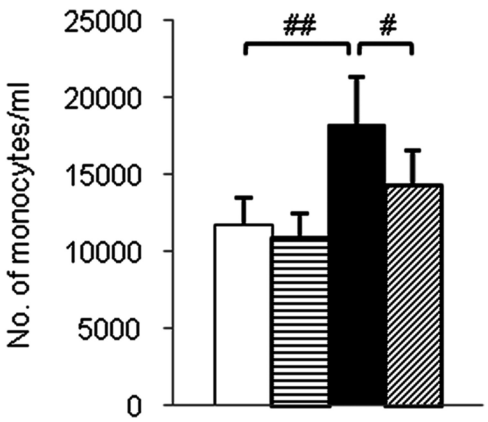

Figure 6. Effect of $1,25(OH)_2D_3$ on chemotactic migration of peritoneal macrophages toward islet medium Islets were exposed in vitro for 72 hours to the following: open bar, control medium; shaded bar, 10^{-8} M $1,25(OH)_2D_3$; solid bar, 50 U/mL IL-1β and 100 U/mL IFN-γ; and hatched bar, 10^{-8} M $1,25(OH)_2D_3$, 50 U/mL IL-1β, and 100 U/mL IFN-γ. Data are presented as means ± SEM (n = 7 independent experiments; #, $P < .01$; ##, $P < .02$).

To understand the mechanisms involved in these beneficial effects of $1,25(OH)_2D_3$, we performed microarray analysis on murine islets exposed to cytokines in the pres-

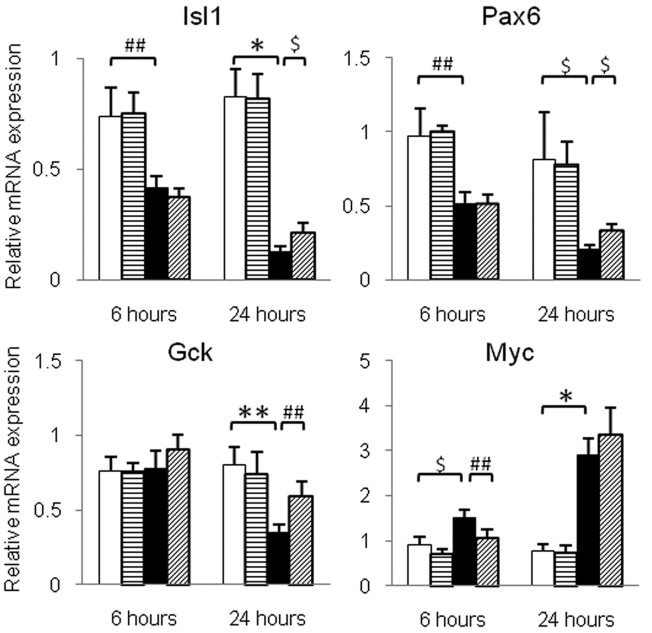

Figure 7. Effect of 1,25(OH)$_2$D$_3$ on mRNA expression of *Gck*, *Isl1*, *Pax6*, and *Myc* in cytokine-exposed murine islets Islets were exposed in vitro for 6 and 24 hours to the following: open bar, control medium; shaded bar, 10^{-8} M 1,25(OH)$_2$D$_3$; solid bar, 50 U/mL IL-1β and 100 U/mL IFN-γ; and hatched bar, 10^{-8} M 1,25(OH)$_2$D$_3$, 50 U/mL IL-1β, and 100 U/mL IFN-γ. Data are presented as means ± SEM (n = 4–7 independent experiments; *, $P < .001$, **, $P < .002$; ##, $P < .02$; $, $P < .05$).

ence or absence of 1,25(OH)$_2$D$_3$. We confirm the dramatic alterations taking place in β-cells exposed to inflammatory cytokines that ultimately result in β-cell dysfunction and death, and the data are fully in line with previous reports studying rat or human islets or rodent-derived β-cell lines (21–23). Integrated signaling cascades are affected by the inflammatory cytokines, some of which may launch pathways that are crucial in the dialogue between the β-cell and the immune system (3). Importantly, this analysis indicated that nearly 4000 genes are affected by cytokines after 6 and 24 hours of exposure. 1,25(OH)$_2$D$_3$ counteracted the effects of cytokines on nearly 250 genes. These modified genes belong to functional groups involved in immune response, chemotaxis, cell death, and pancreatic β-cell function/phenotype, providing a mechanistic explanation for the beneficial effects of the hormone in the context of cytokine-induced β-cell dysfunction and death.

NF-κB has been described as a "master switch" for the gene networks regulating cytokine-induced β-cell dysfunction and death and is believed to be proapoptotic in rodent β-cells in vitro (24, 25) and in vivo (26). It is still controversial whether this is also the case in human β-cells, with some studies showing that NF-κB modulates key proapoptotic pathways (27, 28), whereas others suggest a less relevant role for this transcription factor (29). The ability of 1,25(OH)$_2$D$_3$ to decrease several NF-κB signaling members and especially the binding activity of p52 subunits and thus oppose the induction of NF-κB-related genes by IL-1β and IFN-γ suggests that 1,25(OH)$_2$D$_3$ may protect islets against apoptosis through effects on NF-κB signaling. Two of the affected genes, *p52* and *IKKϵ*, have traditionally been considered to be part of the noncanonical NF-κB pathway, which is triggered by developmental stimuli and regulates cellular processes such as proliferation and survival (30–32). Cross talk between the canonical and noncanonical pathways is believed to take place (30). In addition, p52$^{-/-}$ mice display an autoimmune phenotype (33), whereas IKKϵ has been reported to play a critical role in the antiviral response triggered by IFN (34). Previous reports that 1,25(OH)$_2$D$_3$ down-regulates *p65/RelA* and *p52* in human islets (13) and inhibits nuclear translocation of NF-κB complexes in murine islets (35) and RINm5F cells (12) support a role for NF-κB signaling in 1,25(OH)$_2$D$_3$-mediated protection of islets. The reported role for the antiapoptotic A20 protein and for Fas receptor in this protective effect of 1,25(OH)$_2$D$_3$ in human islets (12, 13), however, could not be confirmed in the present study. Again species differences, differences in cytokine cocktail, or differences in pre- vs coexposure of cytokines and 1,25(OH)$_2$D$_3$ may explain these discrepancies.

A group of molecules that has been identified as central in the dialogue between β-cells and the immune system are chemokines, which are produced by the β-cells under inflammatory attack (3, 36). These chemokines are the gene class most markedly affected by 1,25(OH)$_2$D$_3$ in the present study, an observation in line with previous reports (10, 35, 37). Importantly, our data significantly increases the number of known IL-1β- and IFN-γ-induced cytokines and chemokines the expression of which is reversed by 1,25(OH)$_2$D$_3$, and we could confirm a similar regulation in human islets. This, together with the observed counteractive effect of 1,25(OH)$_2$D$_3$ on migration of monocytes toward islet supernatant, suggest that 1,25(OH)$_2$D$_3$ may prevent or attenuate insulitis in T1D. In support of this concept, in vivo studies have documented that pretreatment with 1,25(OH)$_2$D$_3$ or an analog reduces T cell recruitment to the islets of prediabetic nonobese diabetic (NOD) mice or NOD.SCID mice transferred with CD4$^+$ and CD8$^+$ T cells from recently diabetic NOD mice (10, 35).

Further, and in agreement with previous observations in cytokine-treated rat β-cells (23), we observed that cytokines induce a decline in the expression of genes involved in glucose and lipid metabolism as well as a decreased expression of genes encoding various hormone and growth factor receptors, thus contributing to β-cell dysfunction. 1,25(OH)$_2$D$_3$ partially restores the expres-

sion of key β-cell transcription factors, like *Pax6*, *NeuroD1* and, to a minor extent, *Isl1*. These genes are important not only for development of the endocrine pancreas and β-cells but also for maintaining the function of mature β-cells (38–42). There is evidence that β-cells regenerate during T1D (43). However, such regeneration would be limited by the continuous autoimmune attack involving activation of notch signaling and consequent dedifferentiation of β-cells. According to the present data, such effects may be partially antagonized by $1,25(OH)_2D_3$ through a decline in the expression of the notch ligand *Dll1* and the downstream target *Hes1*. Similar effects were seen on the expression of *Myc* and the target gene *Id2*, which encode proteins that prevent binding of transcription factors such as NeuroD1 to target genes (44, 45).

Several genes involved in glucose metabolism as well as transcription factors playing a role in maintaining a differentiated β-cell phenotype are altered by cytokines and partially normalized by $1,25(OH)_2D_3$. These may be responsible for the underlying mechanisms resulting in the improved GSIS by $1,25(OH)_2D_3$. As such, the capacity of $1,25(OH)_2D_3$ to reverse the decline in expression levels of *Gck* as well as the pyruvate dehydrogenase component *Pdha1* may contribute to ameliorate islet function during exposure to proinflammatory cytokines. This is supported by a previous observation of impaired GSIS in islets isolated from a mouse model of β-cell specific PDHA1 deficiency (46). In the second and more prolonged phase of insulin release, other factors than regulation of ATP-dependent potassium channels by cytosolic ATP levels have been implicated including pyruvate cycling. IDH1 catalyzes one of the pyruvate cycling pathways in which cytosolic isocitrate is oxidized to α-ketoglutarate, which can then reenter the mitochondrial Krebs cycle. The observed early normalization (6 hours) of *Idh1* expression by $1,25(OH)_2D_3$ in cytokine-exposed islets may contribute to improve GSIS, because it has previously been demonstrated that suppression of *Idh1* expression by small interfering RNA in INS-1 cells and rat islets impairs GSIS (47). On the other hand, overexpression of *Ldha*, which is usually expressed at low levels in β-cells to ensure that pyruvate is primarily channeled into aerobic mitochondrial metabolism (48), has been shown to cause a decline in GSIS in mouse insulin-producing MIN6 cells (49). An enhanced expression of *Ldha* is exactly what we observe following 24 hours of exposure to IL-1β and IFN-γ. Interestingly, combined treatment with $1,25(OH)_2D_3$ confers a partial protection against this cytokine-induced modification.

The present study is a follow-up of a previous study, in which we described the effects of $1,25(OH)_2D_3$ on whole murine islets without the concurrent exposure to cytokines (50). Major findings were that $1,25(OH)_2D_3$ altered the expression of genes related to cell cycle and growth as well as several ion/solute transport genes. Of particular interest, a potential improvement of insulin secretion by $1,25(OH)_2D_3$ was suggested by modulatory effects on genes involved in cytoskeletal organization and intracellular trafficking as well as other genes previously linked to insulin release. Compared with the present study, it appears that $1,25(OH)_2D_3$-driven gene-regulatory events observed during cytokine exposure are dependent on cytokine signaling because the most important $1,25(OH)_2D_3$-target genes during cytokine exposure differ from those modified by $1,25(OH)_2D_3$ under basal condition. Overall, our current and previous data suggest that any major beneficial effects of superphysiological $1,25(OH)_2D_3$ levels will become apparent only during stressful conditions such as exposure to cytokines in T1D.

Early (6 hours) and late (24 hours) time points were chosen for islet culture and subsequent gene expression analysis in order to identify primary and secondary targets of $1,25(OH)_2D_3$ in the setting of cytokine exposure. Although an early and sustained induction of NF-κB related genes was observed during cytokine exposure, we detected only late counteractive effects by $1,25(OH)_2D_3$, affecting preferentially genes related to apoptosis. It is thus plausible that $1,25(OH)_2D_3$ protects against apoptosis through the late inhibition of NF-κB signaling. In contrast, our data suggest that normalizing actions of $1,25(OH)_2D_3$ on chemokine and cytokine expression are early and potentially direct events, thus suggesting they may occur, at least in part, independently of NF-κB signaling. We also observed an early, but minor, normalization by $1,25(OH)_2D_3$ cotreatment of inducible NOS/Nos2 gene expression. Based on these findings and previous observations by Riachy et al (12), this early inhibition of iNOS expression may contribute to the observed effects of $1,25(OH)_2D_3$ cotreatment on β-cell transcription factors, glucose metabolic genes, and restoration of insulin release as well as protection against apoptosis. The inhibition by cytokines and normalization by cotreatment of genes related to glucose metabolism and to exocytosis, intracellular trafficking, and cytoskeleton-related genes were present either exclusively after 24 hours or with stronger signals than at the 6-hour time point. Thus, these events are most likely secondary to the effects of other genes such as the mentioned transcription factors.

In conclusion, we demonstrate that $1,25(OH)_2D_3$ exerts partial protection against cytokine-induced islet cell death and completely restores insulin release in both human and murine islets following cytokine treatment.

A new theme emerging from our work is that $1,25(OH)_2D_3$ ameliorates cytokine-impaired expression

of metabolically relevant genes such as glucokinase as well as transcription factors involved in maintenance of a differentiated β-cell phenotype, providing an underlying explanation for the observed protection by $1,25(OH)_2D_3$ against cytokine-mediated decrease in GSIS. Moreover, our results point to a major role of the NF-κB-signaling pathway in the protection by $1,25(OH)_2D_3$ against cytokine-mediated apoptosis. Finally, the most significant effect of $1,25(OH)_2D_3$ was to antagonize the induction of a high number of chemokines and proinflammatory cytokines in islets exposed to IL-1β and IFN-γ, both in human and mouse islets, associated with a decreased migration of monocytes toward supernatant of cytokine-$1,25(OH)_2D_3$-treated islets. These effects likely alter the dialogue between the β-cell under inflammatory attack and the immune system and may thus help to decrease islet infiltration during early stages of diabetes development and contribute to prevent β-cell death in vivo. Although additional confirmations on protein expression levels would increase the significance, the present findings strengthen the basis for the use of $1,25(OH)_2D_3$ in the prevention or treatment of T1D, not only via its reported tolerogenic actions on immune cells (51), but also, as shown here, by directly acting on the β-cells.

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